International Immunopharmacology xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Anti-inflammatory activity of flavonoids in Nepalese propolis is attributed to inhibition of the IL-33 signaling pathway

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ARTICLE INFO

Article history:
Received 7 October 2014
Received in revised form 23 December 2014
Accepted 12 January 2015
Available online xxxx

Keywords:
Propolis
Flavonoid
IL-33
NF-κB
IKK
Anti-inflammatory activity

ABSTRACT

Propolis has been used in folk medicine to improve health and prevent inflammatory diseases; however, the components that exhibit its anti-inflammatory activity remain unknown. We herein investigated the effects of flavonoids isolated from Nepalese propolis on the IL-33 signaling pathway to clarify the anti-inflammatory mechanism involved. Of the 8 types of flavonoids isolated from Nepalese propolis, 4 types of compounds, such as 3′,4′-dihydroxy-4-methoxydalbergione, 4-methoxydalbergion, cearoin, and chrysin, markedly inhibited the IL-33-induced mRNA expression of inflammatory genes including IL-6, TNFα and IL-13 in bone marrow-derived mast cells (BMMC). These four flavonoids also inhibited the IL-33-induced activation of nuclear factor κB (NF-κB), which was consistent with their inhibitory effects on cytokine expression. The effects of these flavonoids are attributed to inhibition of IL-33-induced activation of IKK, which leads to the degradation of IκBα and nuclear localization of NF-κB. On the other hand, other flavonoids isolated from Nepalese propolis, such as isoliquiritigenin, plathymenin, 7-hydroxyflavanone, and (+)-medicarpin, had no effect on the IL-33 signaling pathway or cytokine expression. Therefore, these results indicate that 3′,4′-dihydroxy-4-methoxydalbergione, 4-methoxydalbergion, cearoin, and chrysin are the substances responsible for the anti-inflammatory activity of Nepalese propolis.

1. Introduction

Interleukin-33 (IL-33) is a member of the IL-1 cytokine family, which includes IL-1 and IL-18 [1]. It functions as a central regulator in several allergic disorders, such as asthma, allergic rhinitis, allergic conjunctivitis, rheumatoid arthritis, inflammatory bowel diseases (IBD), and atopic dermatitis. Previous studies have suggested that IL-33 may function as an alarmin, and mature IL-33, which is secreted from necrotic cells, has been shown to stimulate the immune system during allergic inflammation [2–7]. IL-33 has been detected in the bronchoalveolar lavage fluid (BALFs) of asthma patients [8] and IL-33 levels were reported to be higher in patients with systemic sclerosis and correlated positively with the extent of skin sclerosis and severity of pulmonary fibrosis [9]. Pastorelli et al. also reported a correlation between IL-33 mRNA levels and disease severity in model mice of IBD [10], suggesting that IL-33 may be involved in the pathogenesis of allergic disorders. Mast cells, one of the major effector cell populations that mediate allergies, express high levels of ST2L, which is a member of the IL-1 receptor (IL-1R)/Toll-like receptor (TLR) superfamily that was identified as a receptor for IL-33 by Schmitz et al. [11]. In allergic inflammatory responses, IL-33 induces the secretion of not only pro-inflammatory mediators such as IL-6, IL-1, TNFα, and CCL2/MCP-1, but also Th2-associated cytokines such as IL-5 and IL-13 from mast cells [12–14]. Furthermore, IL-33 has been shown to increase surface IgE levels and trigger the degranulation of mast cells [15]. As the involvement of IL-33 in various allergic inflammatory diseases becomes clear, these findings open a new perspective for the treatment of allergic diseases by targeting the IL-33/ST2L signaling pathway.

IL-33 induces the activation of nuclear factor-kappa B (NF-κB), which is a critical transcription factor in inflammation through ST2L [11]. Once stimulated with IL-33, ST2L recruits IL-1R accessory protein (IL-1RAcP) to its signaling complexes in order to activate downstream signaling pathways [16,17]. IL-33–ST2L axis utilizes a common signaling pathway with the IL-1R/TLR superfamily. Thus, myeloid differentiation factor 88 (MyD88), IL-1R associated kinase-1 (IRAK-1), and TNF receptor associated factor 6 (TRAF6) are recruited to ST2L [18,19]. The signaling complexes of ST2L induce the activation of IκB kinase (IKK),

Abbreviations: BALF, bronchoalveolar lavage fluid; BMMC, bone marrow-derived mast cells; FBS, fetal bovine serum; IBD, inflammatory bowel diseases; IκB, inhibitory of NF-κB; IKK, IκB kinase; IL, interleukin; IL-1RAcP, IL-1R accessory protein; IL-1R/TLR, IL-1 receptor/Toll-like receptor; IRAK-1, IL-1R associated kinase-1; LPS, lipopolysaccharide; MyD88, myeloid differentiation factor 88; NF-κB, nuclear factor-kappa B; TNFα, tumor necrosis factor alpha; TRAF6, TNF receptor-associated factor 6; WST-1, water-soluble tetrazolium-1.

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http://dx.doi.org/10.1016/j.intimp.2015.01.012
1567-5769/

and initiate well-known events for the activation of NF-κB, such as the phosphorylation of inhibitory of NF-κB (IκB), proteasomal degradation of phosphorylated IκB, and nuclear translocation of NF-κB. Activated NF-κB then binds to specific sequences in the promoter or enhancer regions of target genes that are involved in inflammatory reactions [20–22].

Since it exhibits numerous biological and pharmacological properties, such as immunomodulatory and anti-inflammatory activities, propolis has been used in folk medicine as a supplement to the daily diet to improve health and prevent inflammatory diseases; however, little is known about its mechanism of action in immunity and inflammation [23–25]. We previously fractionated the extracts of Nepalese propolis and identified several flavonoids including a novel flavonoid, 3′,4′-dihydroxy-4-methoxydalbergione [26,27]. So far, a numerous studies reported the inhibitory effects of flavonoids on LPS-TLR4 signaling pathway, although their detailed inhibitory mechanism is unclear. Signaling cascade induced by LPS has been clarified to utilize the common signaling molecules with IL-33, such as IRAK and TRAF6 [28]. However, it is still unclear whether flavonoids affect the IL-33 signaling pathway, and how these flavonoids exhibit the biological effects.

In the current study, we focused on the effects of 8 kinds of flavonoids, including (1) isoliquiritigenin, (2) plathymenin, (3) 7-hydroxyflavanone, (4) chrysin, (5) 3′,4′-dihydroxy-4-methoxydalbergione, (6) 4-methoxydalbergion, (7) cearoin, and (8) (+)-medicarpin, isolated from Nepalese propolis on IL-33-induced cytokine expression using bone marrow-derived mast cells (BMMC). We also investigated the effects of each flavonoid on the IL-33 signaling pathway in order to clarify the mechanism underlying the anti-inflammatory activity of propolis.

2. Materials and methods

2.1. Antibodies and reagents

A fraction containing flavonoid mixture was prepared from propolis from Chiwan, Nepal, by extraction using methanol and water. Eight kinds of flavonoids, isoliquiritigenin, plathymenin, 7-hydroxyflavanone, chrysin, 3′,4′-dihydroxy-4-methoxydalbergione, 4-methoxydalbergion, cearoin, and (+)-medicarpin, were then isolated as previously reported. Purity of each flavonoid was determined by HPLC analysis, and confirmed to be more than 95% [26,27]. Recombinant murine IL-3, IL-33 and human TNFα were purchased from PEPROTECH (Rocky Hill, NJ, USA). Antibodies recognizing NF-κB p65, Lamin B, IκBα, IKKγ, and β-actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Horseradish peroxidase-conjugated anti-rabbit, anti-goat, and anti-mouse polyclonal IgG antibodies were purchased from Dako-Japan (Tokyo, Japan).

2.2. Preparation of bone marrow-derived mast cells (BMMC) and cell cultures

BMMC were prepared according to procedures established by Razin et al. [29]. Bone marrow cells (1×10^6/mL) from 6- to 8-week-old C57BL/6 mice (Sankyo Laboratory Service, Tokyo, Japan) were cultured with DMEM (Nacalai Tesque, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) (BioWest, Nuaillé, France), 100 units/mL penicillin (Nacalai Tesque), 100 μg/mL streptomycin (Nacalai Tesque), 0.1 M non-essential amino acids (Nacalai Tesque), and IL-3 (5 ng/mL). After a 4-week culture, viable cells were stained with acidic toluidine blue and confirmed to have differentiated into mast cells. NIH-3T3 stably expressing the NF-κB-dependent luciferase reporter plasmid was established as previously described [30]. NIH-3T3/pNF-κB-Luc cells were cultured with DMEM (Nacalai Tesque) supplemented with 10% FBS, 100 units/mL penicillin (Nacalai Tesque) and 100 μg/mL streptomycin (Nacalai Tesque).

2.3. WST-1 assay

The cell proliferation reagent water-soluble tetrazolium-1 (WST-1) (Roche Applied Science, Indianapolis, IN, USA) was used to detect the metabolic activity of cells. BMMC (1×10^5 cells/100 μL) were seeded in 48-well plates. Cells were pretreated with various concentrations of propolis-derived flavonoids for 1 h prior to the stimulation with IL-33 (10 ng/mL) for 24 h. 10 μLWST-1 was then added to the culture media and cells were incubated with the reagent for 2 h in a 37 °C, 5% CO_2 environment. Absorbance was read using the microplate reader Infinite M1000 (Tecan Group Ltd. Tokyo, Japan) at 450 nm and 690 nm.

2.4. ELISA (enzyme-linked immunosorbent assay)

BMMC (1×10^6 cells) were cultured in a 24-well plate and pretreated with various concentrations of propolis-derived flavonoids for 1 h at 37 °C prior to the stimulation of IL-33 (10 ng/mL) for 18 h. The culture supernatants were collected, and the amounts of IL-6 and IL-13 in the supernatants were determined using immunoassay kits (eBioscience, San Diego, CA, USA).

2.5. RNA isolation and RT-PCR (reverse transcriptase-polymerase chain reaction)

RNA was prepared using an RNA purification kit (Qiagen, Hilden, Germany). RT was performed using an oligo $(dT)_{20}$ primer and 1 μg total RNA for first-strand cDNA synthesis, as described previously [19]. Quantitative real-time PCR was performed using an iCycler detection system (Bio-Rad, Berkeley, CA, USA). PCR was performed in a 25 μL volume with KAPA SYBR® FAST qPCR Kits (KAPA Biosystems, Wilmington, MA, USA). The PCR primer sequences were as follows: IL-6, 5′-CCAGAG ATACAAAGAAATGATGG-3′ (forward) and 5′-ACTCCAGAAGACCAGAGG AAAT-3′ (reverse); IL-13, 5′-CAGTCCTGGCTCTTGCTT-3′ (forward) and 5′-AGGCCATGCAATATCCTC-3′ (reverse); TNFα, 5'-TACTGAACTTCGG GGTGATCGGTCC-3' (forward) and 5'-CAGCCTTGTCCCTTGAAGAGAA CC-3' (reverse); GAPDH, 5′-ACTCCACTCACGGCAAATTC-3′ (forward) and 5′-CCTTCCACAATGCCAAAGTT-3′ (reverse).

2.6. Immunoblotting

Cells were washed with ice-cold PBS and lysed in Nonidet P-40 lysis buffer (50 mM Tris–HCl, pH 8.0, 120 mM NaCl, 1 mM EDTA pH 8.0, 0.5% Nonidet P-40, 10 mM β-glycerophosphate, 2.5 mM NaF, 0.1 mM Na_3VO_4) supplemented with protease inhibitors. To prepare nuclear extracts, cells were lysed in buffer A (10 mM HEPES-KOH (pH 7.8), 10 mM KCl, 0.1 mM EDTA, 0.1% Nonidet P-40, 1 mM DTT, 0.5 mM PMSF, 2 μg/mL aprotinin, 2 μg/mL pepstatin, 2 μg/mL leupeptin). Nuclei were then isolated by centrifugation at 5,000 r.p.m for 2 min. Isolated nuclei were lysed in Nonidet P-40 lysis buffer and homogenized using the ultrasonic homogenizer VP-50 (TAITEC, Japan). Nuclear extracts were then centrifuged at 15,000 rpm for 15 min at 4 °C and the supernatant was mixed with Laemmli's sample buffer. Denatured samples were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA). Membranes were probed using the designated antibodies and visualized with the ECL detection system (GE Healthcare, Little Chalfont, UK) [19]. The intensity of each band was quantified by Image-J software. To show the relative amount of IκBα, the band intensity of IκBα was normalized with that of β-actin.

2.7. Transfections and luciferase assay

BMMC (6×10^5) were transfected with 1.2 μg of pNF-κB-Luc (Promega, Madison, WI) and 0.3 μg of pRL-TK (Promega) using the Neon® Transfection System (Life Technologies, Waltham, MA). Transfected cells were pretreated with DMSO (0.1%) or 30 μM of the propolis-derived flavonoids for 1 h prior to the stimulation with IL-33

Please cite this article as: Funakoshi-Tago M, et al, Anti-inflammatory activity of flavonoids in Nepalese propolis is attributed to inhibition of the IL-33 signaling pathway, Int Immunopharmacol (2015), http://dx.doi.org/10.1016/j.intimp.2015.01.012